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Molecular genetic analysis of the model organism *Caenorhabditis elegans* was used to identify and study mechanisms of action of negative regulators of tyrosine kinase/*ras* mediated signal transduction that are candidate tumor suppressors. A homolog of the proto oncogene *cbl,* SLI-1, inhibits Ras activation by the epidermal growth factor receptor homolog LET-23. The domain of human c-cbl most highly conserved functions in *C. elegans* when substituted for the homologous region of SLI-1. Proteins that interact with this region of SLI-1 have been identified by a "two-hybrid" screen in yeast. ROK-1, a novel tyrosine kinase, has been characterize. Both SLI-1 and ROK-1 have proline-rich domains that are necessary for their function. New screens for additional negative regulators have identified at least three genes that are now being analyzed. Human homologs of ROK-1 are now being sought.

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Paul W Stewart 12/22/97

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Introduction

An important short-term goal of breast cancer research is to identify candidate genes for identifying pre cancerous cells and cancerous cells. Ideally, pre cancerous or cancerous cells could be characterized by a set of molecular markers. These markers would best be chosen from the set of genes altered during early stages of precancer formation. It is also crucial to understand the cellular regulatory pathways in which these genes act. *C. elegans* molecular genetics provides a facile model system with which to identify and to examine gene interactions *in vivo* [reviewed by Sternberg, 1993]. For example, it has been clearly shown that the *C. elegans* ras protein LET-60 acts downstream of EGF-receptor homolog LET-23 [Han & Sternberg, 1990; Aroian et al. 1990], that the adaptor protein SEM-5 acts between LET-23 and ras [Clark et al., 1992; Katz, et al., 1996] and that the LIN-45 raf protein acts downstream of LET-60 ras [Han et al., 1993]. The ligand for LET-23 is likely LIN-3, an EGF-like growth factor [Hill and Sternberg, 1992; Katz et al., 1995]. This universal signaling pathway is the target of many mutations contributing to oncogenesis in humans. Overexpression or activation by mutation of LIN-3, LET-23, LET-60 results in excessive vulval differentiation; thus activation of the homologous genes leads to cancer in humans and vulval differentiation in nematodes. Therefore, negative regulators of vulval differentiation defined by loss-of-function mutations that lead to excessive signaling are analogous to tumor suppressor loci. During normal development, the gonadal anchor cell induces precisely 3 of 6 vulval precursor cells (VPCs) to divide and differentiate into vulval tissue. Even if 10% of experimental animals have excessive vulval tissue -- indicative of hyperactivation of the ras pathway -- we can detect it with confidence.

Using the powerful genetics of *C. elegans*, we and others have identified multiple pathways of negative regulation of LET-23 mediated signaling have been identified. We are carrying out genetic screens that can identify additional genes involved in negative regulation, either in known pathways or in new ones. Our genetic studies have identified several negative regulators of LET-23-mediated signaling in *C. elegans* [reviewed by Sternberg, 1994]: SLI-1 [Jongeward et al., 1995; Yoon et al., 1995], UNC-101 [Lee et al., 1994], ROK-1, SLI-2 and ROT-1 [see details below], LIN-15 [Ferguson & Horvitz, 1989; Huang et al., 1994]. In general, these negative regulators are redundant, such that elimination of any one has no effect on the normal signal transduction. In the absence of two regulators, excessive vulval differentiation occurs. These genetic properties are similar to the synergistic action of oncogenic mutations, where several mutations are necessary for a phenotypic change. However, such synthetic mutations are difficult to study except in genetically facile organisms such as *C. elegans.*

If we can identify many loci possibly involved in the analogous processes in *C. elegans*, and identify human homologs, this will help human geneticists in several ways. First, this will provide candidate genes for the positional cloning of tumor suppressor loci defined by human cancer genetics. Second, this will provide molecular probes with which to examine tumorous tissue for alterations. The sooner we can identify the many potential tumor suppressor loci, the more effectively that analysis of the role of tumor suppressor mutation in breast cancer initiation and

progression can be assessed. Current technology easily allows parallel processing of samples, and is thus limited by the number of molecular probes. Lastly, we can link together tumor suppressor genes in functional pathways, much as we have been able to do for the LET-23-mediated pathway. If there are multiple pathways of tumor suppressor gene action, then we need to know how to intervene in each one.

One implication of multistep carcinogenesis is that synergism occurs between mutations. The genetics of the negative regulators that we have identified is analogous: mutation of, for example, SLI-1 or ROK-1 alone causes no defect, yet inactivation of both leads to increased signaling. The roles of such apparently redundant genes are difficult to study except in powerful genetic systems; *C. elegans* vulval differentiation provides such a system.

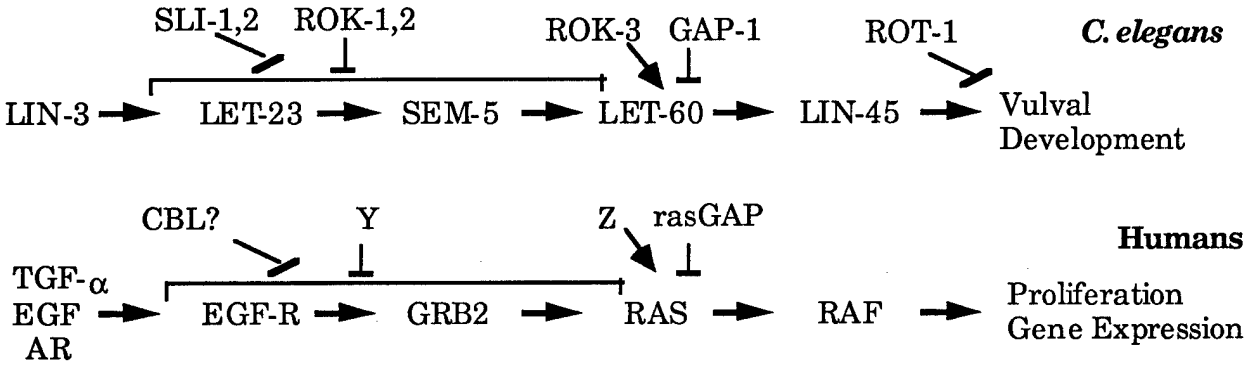


Figure 1. Pathway for the major EGF-R signaling pathway in humans and the analogous LET-23-mediated signaling pathway in *C. elegans*. Arrows indicated positive regulation; bars represent negative regulation. Other components are known. The universality of the side branches is yet to be shown rigorously.

The oncoprotein cbl has continued to be linked to signaling in many human cell types. Its physiological role remains elusive, and our molecular genetic studies of SLI-I are designed to help understand the function(s) of this new family of proteins. The roles of cytoplasmic tyrosine kinases in signaling and its regulation is another major area of research. We have discovered that *rok-1* encodes such a tyrosine kinase, and we have begun to examine its mechanism of action as well. Lastly, regulation at the level of the nuclear targets of tyrosine kinase signaling is yet another area of uncertainty, and analysis of the new gene *rot-1* may help clarify this area.

The goal of this project is to identify potential tumor suppressor genes and link them into functional pathways with each other, and with known proto-oncogenes and tumor suppressor genes. *C. elegans* vulval differentiation provides a facile model system with which to study EGF-receptor/*c-neu*-mediated signal transduction and its regulation. The *C. elegans sli-1, sli-2* and *rok-1* genes negatively regulate LET-23-ras signal transduction. We are using *C. elegans* molecular genetics to study and clone these genes in *C. elegans*, and will use molecular biology to clone their human homologs.

Body

The specific goals of the project are as follows.

1. **Analyze SLI-1 function** in *C. elegans* through molecular genetics.
2. **Molecularly clone *sli-2*.**
3. **Molecularly clone *rok-1*.**
4. **Identify and clone additional genes acting in concert with *sli-1*, *sli-2*, and *rok-1***
5. **Examine the functional interactions of *sli-1*, *sli-2*, *rok-1* in regulating other conserved signaling pathways.**
6. **Clone human *sli-2*, *rok-1*, and newly identified genes from human breast tissue libraries** to generate reagents with which to test the hypothesis that these are novel tumor suppressor loci.
7. **Test the functional homology of *c-cbl* and *sli-1*** by introducing the human cDNA into transgenic nematodes defective in *sli-1*.

In the following, progress on these goals is described. Section headings labeled in italics are methods.

1. Molecular genetics of *sli-1*, *C. elegans* homolog of cbl and cbl-b.

We identified the *sli-1* locus as a negative regulator of LET-23, the *C. elegans* homolog of EGF-R/c-neu/HER3/HER4, using extragenic suppressor analysis [Jongeward et al., 1995]. We cloned the *sli-1* locus by correlating genetic and physical maps and rescuing a *sli-1* mutant in transgenic nematodes [Yoon et al., 1995]. A 10.5 kilobase genomic fragment has the ability to provide all known functions of *sli-1* in transgenic animals, i.e., it rescues the suppression phenotype of *let-23*.

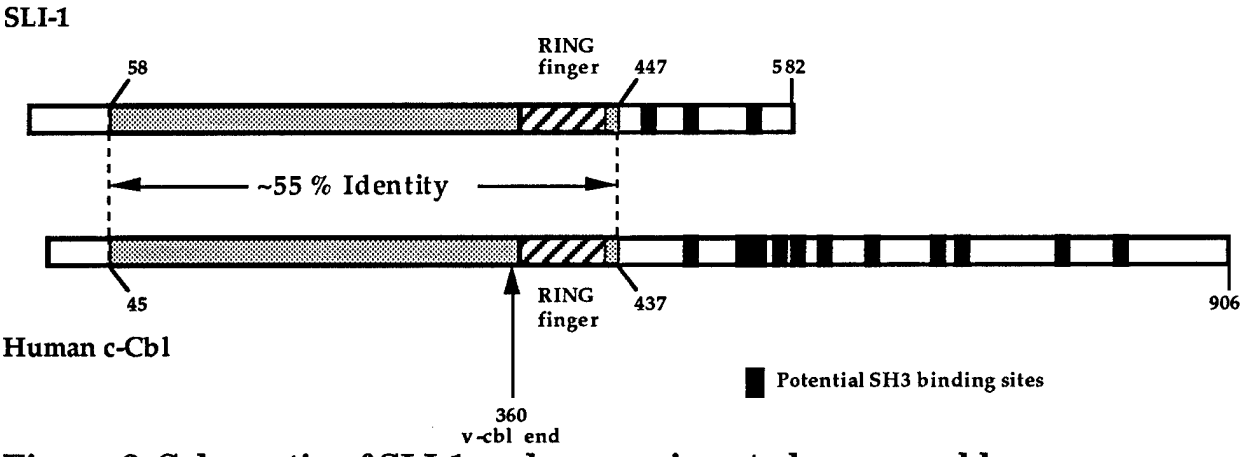


Figure 2. Schematic of SLI-1 and comparison to human c-cbl.

c-cbl, cbl-b and *sli-1* each contain a Ring Finger motif [$CX_2C_{9-27}CX_{1-3}HX_2CX_2CX_{6-48}CX_2C$] putative metal binding finger [Lovering et al., 1993]. The truncated, oncogenic form of *v-cbl* is missing the finger, as is the truncated form found in HUT78 T cell lymphoma cells [Blake & Langdon, 1992]. This year we have completed our

analysis of functional domains of SLI-1 in a transgenic minigene system. In this assay, a strain without functional SLI-1 has 2.5 vulval precursor induced by the RAS pathway while a strain with SLI-1 has 0.5. We find that the amino-terminus of SLI-1 is necessary and partly sufficient for its activity. The proline-rich carboxyl terminus and RING finger is necessary for full activity, but individual proline-rich (SH3 binding regions) are redundant. For example, deletion of the proline-rich domain increases vulval induction from 0.5 to 1.0 (p=0.0003, non-parametric Mann-Whitney Test; InStat program). The proline-rich domain interacts with SH3 domain of SEM-5 in a yeast two-hybrid assay. The RING finger is necessary for full activity. A potential myristylation signal at the amino-terminus is not necessary for activity in our assay.

We have completed the structure/function analysis of SLI-1. The final results are summarized in Figure 3. A manuscript describing these studies, along with genetic data on *let-60 ras* suppression and the SLI-1/SEM-5 two-hybrid experiment described below has been written and is soon to be submitted.

Construction of heatshock minigenes

Standard molecular biological techniques were used as described by Sambrook et al. (1989). Site directed *in vitro* mutagenesis was carried out in double stranded DNA according to the method prescribed by Deng and Nickoloff (1992) and with reagents and specific protocols from Clonetech (Palo Alto, CA). The mutagenesis was carried out in the plasmid pCY-D6, a pSK+ vector containing the full-length *sli-1* cDNA inserted into the EcoRI site. A selection primer which changed the novel NotI site in the vector to an NheI site was used in conjunction with mutagenic primers which mutated or removed sequences of varying length within the *sli-1* coding region. Mutagenized *sli-1* cDNA was then digested with SpeI/EcoRV and inserted into the NheI/EcoRV sites of the pPD49.83 and pPD.49.78 nematode heatshock minigene vectors. Mutagenesis was confirmed by restriction mapping and by sequencing. The deletion constructs SLI-1.ΔC, SLI-1.ΔN, SLI-1.ΔNΔC, SLI-1,ΔPro2, and SLI-1.ΔPro3 replace the deleted sequences with an NheI site which codes for alanine and serine in frame with the rest of the *sli-1* construct.

Myristylation site mutagenesis used the previously mutagenized *sli-1* cDNA using a selection primer which changed a unique XhoI site into a ClaI site. The cloning into heatshock vectors followed identical procedures as above. The conserved N-terminal domains of human c-Cbl was PCR amplified from the cDNA in the pUC vector (provided by W. Langdon). The ends of the amplification primers contained in-frame NheI sites. The amplified conserved N-terminal fragment of c-Cbl was then purified, digested and ligated into the NheI site of the SLI-1.ΔN construct in pPD49.83. The directionality of the insert was checked by restriction digests and the sequence of the amplified c-Cbl fragment was verified by DNA sequencing. DNA sequencing was carried out on automated sequencers by the Caltech sequencing facility (Applied Biosystems).

Transgene construction and heat shock protocol

Germline transformation was carried out according to the methods of Mello et al. (1991). Heatshock constructs were injected at 50 ng/μl along with 150 ng/μl of SK+ plasmid and 15 ng/μl of pMH86 (*dpy-20*$^+$ marker) into the germline of *let-23(sy1); dpy-20(e1282); sli-1(sy143)* parental progeny. Independent non-Dpy transformant

lines were maintained and selected for transgene stability. F3+ stable transgenic progeny were selected for egg-lay cohorts and heatshock analysis.

Thirty egg laying young hermaphrodites were picked onto fresh plates, ten per plate. These were then allowed to lay eggs on given plates for one hour before being moved to fresh plates. The transfer of worms was maintained for six consecutive hours. The plates were maintained at 20 °C and then heatshocked together for 30 minutes at 33 °C, 36 hours after the last heatshock cohort. This generated cohorts of worms that had been heatshocked on hourly intervals from 36 to 42 hours after egg lay. This coincides with the early L3 stage vulval induction in the non-Dpy germline-transformed genotype *let-23(sy1); dpy-20(e1282); sli-1(sy143).* The heatshocked plates were returned to 20 °C for 18 to 24 hours before the non-Dpy progeny were scored for vulval induction. A minimum of two independent lines were scored per heatshock construct, usually derived from separate injection events. 15 to 30 worms were scored per one hour time point per stable line. Data from two to three consecutive time points that generated the lowest induction levels in a given experiment were tabulated.

Microscopy. The extent of vulval differentiation was measured by examining vulval anatomy in early to mid L4 stage animals (Sulston and Horvitz, 1977; Sternberg and Horvitz, 1986). Hermaphrodites were placed on 5 % Noble Agar pads and scored with a Plan 100x objective, Nomarski differential interference-contrast optics. 1° and 2° vulval fates were scored as vulval cells. Wild-type=3 VPCs undergoing vulval differentiation/worm=100 % vulval differentiation.

We demonstrated previously that *sli-1(1f)* is unable to suppress severe, null alleles of *let-60*. We tested the interactions of *sli-1* with *sem-5* [Clark et al., 1992]. The severe but non-null allele of *sem-5, n1619*, is strongly suppressed by *sli-1(sy194)*. However, a null allele of *sem-5* is not suppressed.

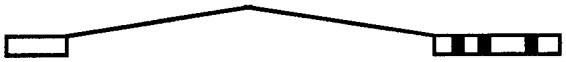
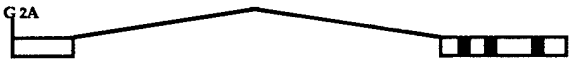
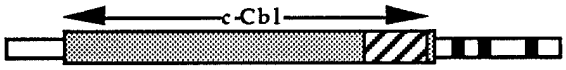
Injected sli-1 heatshock minigene constructs (schematic)	Construct designation	Average number of VPCs undergoing vulval differentiation
No cDNA insert	Control	2.5 (n=58)
	SII-1	0.5 (n=80)
	SII-1.ΔC	1.0 (n=75)
	SII-1.ΔN	2.4 (n=80)
	SII-1.ΔNΔC	2.5 (n=80)
	SII-1.ΔPro2	0.7 (n=55)
	SII-1.ΔExon10	0.9 (n=56)
	SII-1.ΔPro3	0.3 (n=63)
	SII-1.ΔRING	1.5 (n=66)
	SII-1.Δmyr	0.5 (n=67)
	SII-1.ΔCΔmyr	0.9 (n=98)
	SII-1.ΔNΔmyr	2.6 (n=60)
	SII-1.ΔN+CBL.N	1.5 (n=100)

Figure 3. *sli-1* Structure and Function. Individual larval hermaphrodites carrying the transgene depicted are recognized by their non-Dpy phenotype and examined with Nomarski optics to determine the extent of vulval differentiation. A wild-type animal always has three VPCs generating vulval cells. *let-23(sy1)* reduces vulval differentiation to 0.3 VPCs. A *sli-1* mutation restores vulval differentiation to >3 VPCs.

The gray box indicates the domain highly conserved between *sli-1, cbl* and *cbl-b*, a presumed "catalytic" domain. The hatched box indicates the ring finger motif. The black bars represent proline-rich putative SH3-binding domains. Deleted regions are indicated by their angled lines. The last line demonstrates that the conserved N-terminal domain of human CBL can function in *C. elegans*(Task 7) A potential myristylation site (myr) has no obvious function.

Two hybrid analysis of SLI-1.

Two-hybrid protocol. We followed the yeast two-hybrid protocol as described by Fields and Song (1989). Reagents and the yeast strain, CG1945, were from acquired from Clonetech (Matchmaker Kit, Clonetech, Palo Alto, CA). Fragments of SLI-1 and SEM-5 were PCR amplified with primers containing BamHI and PstI restriction sites in the 5' and 3' ends of the cDNAs, respectively. These were then directionally ligated into the corresponding MCS sites of pGBT9 and pGAD424 vectors. Each gene fragment was ligated into both fusion vectors and two-hybrid tests were carried out in reciprocal fusion construct pairs (e.g., pGBT.SEM-5/pGAD.SLI-1 and pGBT.SLI-1/pGAD.SEM-5). Following co-transformation and plating onto SD Trp- Leu- double dropout media, three to four growing colonies per co-transformed line were streaked onto SD Trp- Leu- His- triple dropout media and maintained at 30 °C for two to three days.

The proline-rich C-terminal region of SLI-1, but not its N-terminal region, can interact with SEM-5. Given the suppression of strong mutations of *sem-5* by a *sli-1* mutation, we tested for possible interactions between SLI-1 and SEM-5 using yeast two hybrid assays. Two hybrid fusion constructs containing the C-terminal portion of SLI-1, with and without the RING finger, show robust interaction with full-length SEM-5 based on growth of the yeast colony. The SLI-1 construct that includes both the conserved N-terminal domain and the C-terminal proline-rich domain also interacts with SEM-5 in these assays. By contrast, the N-terminal domains of SLI-1, with or without the RING finger, do not interact with SEM-5 when the C-terminal domain is deleted.

The screen. As an adjunct to genetic screens for SLI-1 partners, we undertook a "two-hybrid" screen in yeast. We constructed plasmids fusing full length SLI-1 to GAL4 DNA binding domain and screened a *C. elegans* cDNA library in pGAD vector, fusing random cDNA inserts to GAL4 activation domain (obtained from R. Barstead pers. comm.).

Of 4.2 x 106 transformants, 590 His+ were picked. 272 were β-galactosidase$^+$ (a second reporter plasmid). 154 of these depend on the bait plasmid; the rest activate transcription on their own and were discarded. 18 of these were analyzed by purifying the prey plasmid and retransformed; 15 of these interact specifically with SLI-1. These 15 clones represent four genes.

clone	chromosome	number obtained	protein product
SIP#1	V	12	2090 aa novel protein
SIP#2		1	ATP synthetase
SIP#3	II	1	1302 aa U6-specific protease
SIP#4	IV	1	349 aa novel

Table 1. Summary of SLI-1 Interacting Proteins (SIPs). For each clone class, the C. elegans chromosome assignment based on comparison to the extensive genomic sequence is indicated, as is the name of the closest relatives based on a BLAST search of non-redundant protein and nucleic acid databases maintained at NCBI. aa, amino acid residues

SIP #1 interacts with N terminus of SLI-1.

We then screened the remaining colonies for genes other than SIP1 using yeast colony hybridization. 124 clones hybridized; 30 did not. We will analyze these 30 clones in the next year. We will also test for physical interaction of SIP#1 and SLI-1 *in vitro*. We will use RNA-mediated gene inactivation [C. Mello, pers. comm.] to test for function *in vivo*.

2. *sli-2*. *sli-2* was isolated as a mutation that failed to complement a *sli-1* mutation. *sli-2(sy262)* is semidominant. As shown in Table 2, we have found that sli-2 suppresses the vulvaless phenotype of *let-23*. We therefore constructed a strain that was doubly heterozygous for *sli-1* and *sli-2* to determine the strength of the interaction. The *let-23* allele *sy1* provides a sensitive genetic background in which to analyze interactions of negative regulators. We therefore analyzed interactions of *sli-1* and *sli-2* in this background. We found no significant difference between *sli-2*/+ and *sli-2*/+ *sli-1*/+ (p=0.0961; Mann-Whitney Test)

A *sli-2* mutation only weakly synergizes with *sli-1* (also see section 5 below). Our hypothesis remains that SLI-2 is a partner of SLI-1. We have just constructed a *sli-2 rok-1* double mutant to test this hypothesis.

genotype			phenotype	conclusion
let-23	*sli-1*	*sli-2*	vulval induction (n)	
+/+	-/-	-/-	**3.1 (40)**	**only weak synergy**
sy1/sy1	+/+	+/+	**0.3 (many)**	**control**
sy1/sy1	+/+	-/+	**3.1 (60)**	***sli-2* is semidominant**
sy1/sy1	-/+	+/+	**0.7 (35)**	
sy1/sy1	-/+	-/+	**3.5 (40)**	
sy1/sy1	+/+	-/-	**4.3 (40)**	***sli-2* suppress *let-23***
sy1/sy1	-/-	-/-	**4.8 (40)**	**only weak synergy**

Table 2. Interactions of *sli-1* and *sli-2*

We have mapped *sli-2* to a genetic interval comprising 1 megabase of DNA. We have prepared YAC clones spanning this region and have begun injecting them into *let-23; sli-2* double mutants to rescue the suppression phenotype of *sli-2.* In addition, we are refining the genetic map position. Progress was slower than anticipated since Charles Yoon finished his Ph.D. thesis prior to cloning *sli-2.* Christopher Lacenere and John DeModena now have this as their top priority experiment.

3. Genetics and molecular cloning of *rok-1*

We identified the *rok-1* (*r*egulator *o*f *k*inase) locus (*IV*) of the nematode *C. elegans* in a genetic screen for new negative regulators of the vulval induction pathway. At 20°C, *rok-1(sy247)* causes essentially no phenotypes in an otherwise wild-type background. However, in combination with mutations in other negative regulator genes, such as *sli-1*, it causes pleiotropic effects including excessive vulval differentiation larval lethality and abnormal spicules. *rok-1* differs in other negative regulators in that it appears to be exclusively on stimulated activity of the signaling pathway. We have almost completed the initial molecular and genetic characterization of *rok-1.* and are preparing a manuscript as we finish the last experiments.

We showed previously that *rok-1(sy247)* is a temperature sensitive mutation wild-type at 20°C but at occasionally multivulva at 25°C (3.1 cells per animal). *rok-1(sy247)* is a strong loss of function mutation. We also examined the spectrum of suppression of vulvaless mutations by *rok-1.* The strong suppression of *sem-5(n1619)* is particularly notable and may indicate that *rok-1* acts at this step. We cloned the *rok-1* locus and found it to encode a single transcript of 3.6 kilobase. Expression of this 3.6 kilobase cDNA from a heat shock promoter is able to rescue the defects associated with the *rok-1(sy247)* mutation. The predicted open reading frame of this transcript encodes a novel cytoplasmic tyrosine kinase of the structure shown in Figure 4. We determined the molecular lesion associated with the *rok-1(sy247)* mutation by standard lab procedures (e.g., Yoon et al., 1995). It changes codon 528 to a termination codon (Q528och). This result confirms that we have indeed cloned the *rok-1* locus.

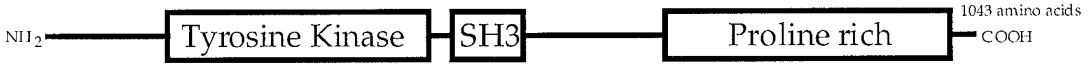


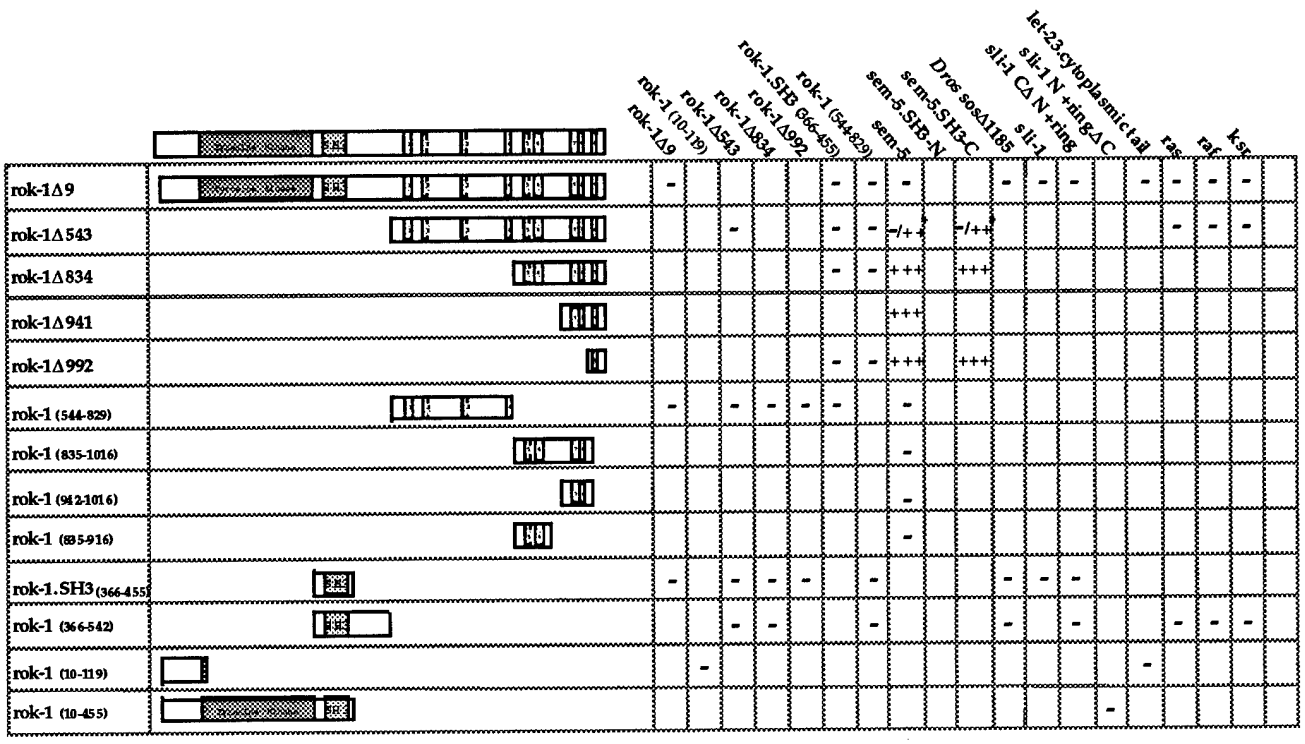
Figure 4. Schematic of ROK-1 protein, which has a similar structure to human Ack and is 50% identical in its tyrosine kinase domain.

Given that the genetic evidence described last year suggests that *rok-1* acts in the LET-23 - LET60 pathway, we sought to determine whether ROK-1's SH3 domain or potential SH3 binding sites in the proline rich C terminal tail interact with SEM-5 or SOS. To do this, we have used the yeast two hybrid system. Since the *C. elegans* homologue of *sos* is not yet cloned we used *Drosophila* SOS. In these assays, we detect interaction between ROK-1's proline rich C terminal and the C terminal SH3 domain of SEM-5. We also detect interaction between *Drosophila*

SOS and the C terminal SH3 domain of SEM-5. However, we detect no interaction between ROK-1's SH3 domain and ROK-1's own proline rich tail or with SOS (Table 4). In addition, we also have failed to detect interaction between the N terminal SH3 domain of SEM-5 and ROK-1; however, there is no positive control for the N terminal domain of SEM-5. ROK-1 fails to interact with RAS, RAF or KSR.

Thus, we have physical and genetic evidence that *rok-1* acts in the receptor tyrosine kinase - *ras* pathway. The *sem-5* mutation *n1619* results in the substitution of a proline for leucine in the N terminal SH3 domain of SEM-5 and produces a protein severely compromised in its functions. *rok-1(sy247)* allows this defective adaptor protein to signal effectively. The physical interaction between ROK-1 and SEM-5 utilizes the C terminal SH3 domain of SEM-5 and is therefore consistent with a model that this interaction leads to negative regulation. The relatively weak suppression of *let-23(sy97)* may indicate that negative regulation by *rok-1* requires the receptor's SEM-5 binding sites. This is also consistent with the model that negative regulation by *rok-1* is mediated through SEM-5. There are two simple models of how the ROK-1:SEM-5 interaction could be used for negative regulation. First, this interaction itself could negatively regulate SEM-5 from binding SOS by competition. Alternatively, this interaction could be a mechanism of recruitment of ROK-1 into the complex which then leads to negative regulation by other means.

Since *rok-1* encodes a novel protein, although one with recognizable domains, it is crucial to determine which domains are important for ROK-1 function. We therefore established a minigene system to facilitate site-directed mutagenesis studies. Approximately 3 kilobases of sequences 51 to *rok-1* coding region was fused to a full length *rok-1* cDNA. A terminator from a standard *C. elegans* vector was included at the 3' end.



PxxP

1: SPPVARAPSQPPSYSQPRPPPRSVS
2: DSRGSLTPTAPPLTA
3: RKDPIPAPRGPVAA
4: KPGEIEEPQQPAESLY
5: QASVSPPPMSPTS
6: ISPAPPPEVTPPLSVR
7: RKAPAPTPVSPAPAG
8: STDQKPPPCRPPKT
9: AGARVAPPVPPKPKV

* in each case interaction was only detected with rok-1Δ543 inserted into pGAD-424; no interaction was observed with rok-1Δ543 inserted into pGBT-9 despite the pGBT: rok-1Δ543 construct being made and tested 3 times from 2 separate PCR readions

Table 4. Two-hybrid analysis.
GBT-9 encodes the GAL-4 DNA binding domain fused in frame with test protein. GAD-424 encodes the GAL-4 transactivation domain fused in frame with test protein. (-) indicates no fusion protein and thus a control construct. + indicates growth of yeast strain and hence protein-protein interaction; - indicates no growth and hence no interaction.

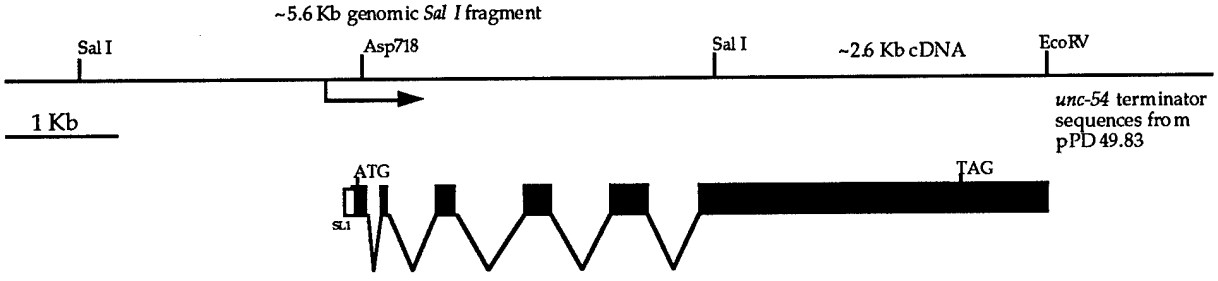


Figure 5. Schematic of rok-1 minigene. Above is the genomic clone, below a full length cDNA.

This minigene rescues the multivulva phenotype of a *rok-1; sli-1* double mutant from 85% (110/130) to 6% (4/72; p <0.0001). Figure 6 depicts the constructs and the extent of rescue. The proline rich region is necessary for ROK-1 function (p =0.57 versus intact clone). The SH3 domain is not necessary for ROK-1 function (p=1). Neither apparently is the kinase. However, since the *sy247* mutation truncates after the SH3 domain, it is likely non-functional since it lacks this important domain, but might complement a minigene encoded protein that has a proline rich domain. We therefore need a protein null mutation of *rok-1*. To obtain this, we screened for new mutations that fail to complement *sy247* under conditions that would recover 25% of mutations (50% loss due to lethality ; 50% loss due to incomplete penetrance). We have two candidates. If these are not nulls, we will use a PCR-based strategy to generate a molecular null. Resolving the null mutation issue is the last experiment prior to publication.

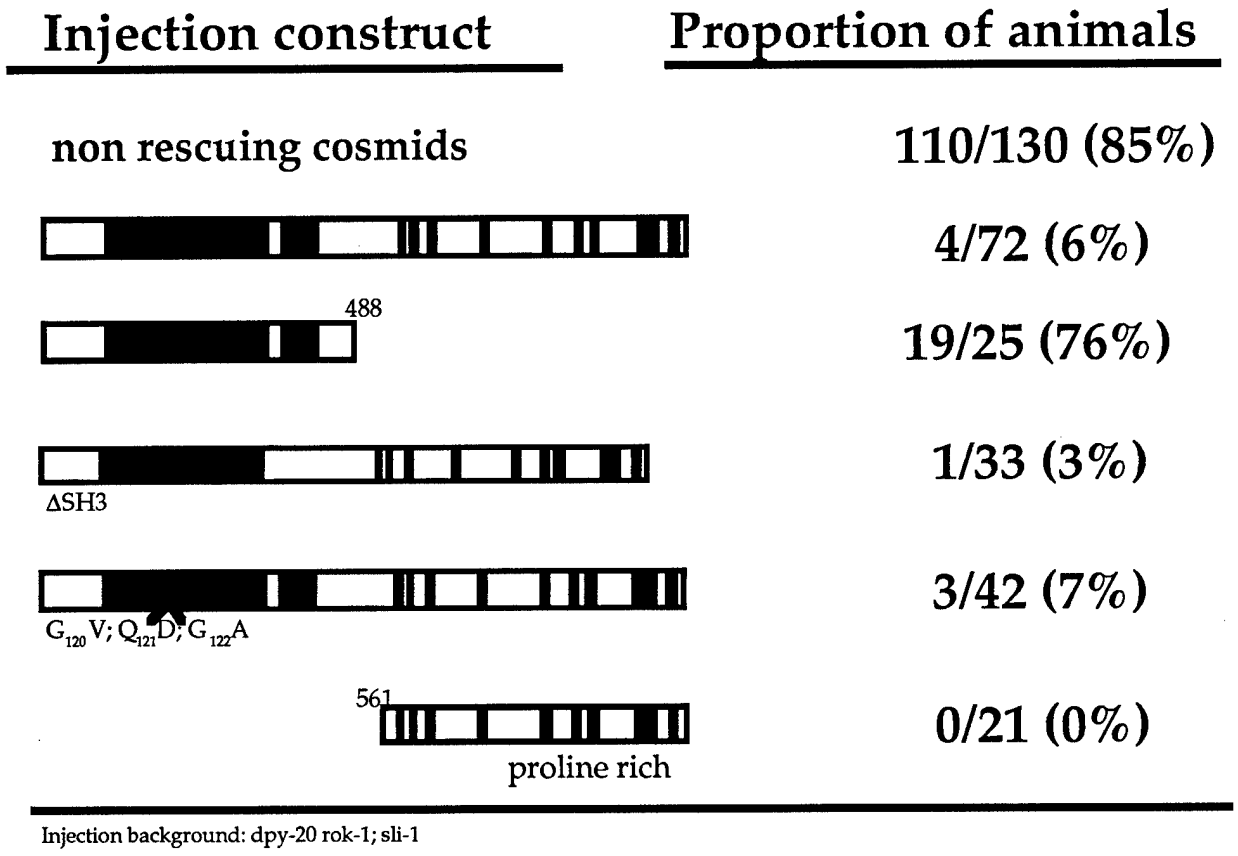


Figure 6. Structure and function analysis of ROK-1.

The proline-rich domain can complement the *rok-1* mutation (position Gln528ochre) that misses the proline-rich domain. Thus, either the Proline-rich domain exerts biological function, or there is *trans* complementation. We are therefore screening for null alleles.

	Average number of VPCs induced	
Genotype	*rok-1(+)*	*rok-1(sy247)*
+/+	3.0 (many)	3.0 (many)
sem-5(n2019)	0.5 (20)	1.28 (20)
sem-5(n2195)	3.0 (20)	3.0 (20)
sem-5(n1619)	0.35 (40)	2.89 (23)
sem-5(ay73am)	0.0 (18)	0.0 (22)

Table 5. New data on *rok-1* suppression of *sem-5*. We observed the extent of vulval differentiation using Nomarski optics as described in (Han and Sternberg, 1990). We observed the vulva differentiation of the animals in their L3 molt stage, when the induced VPCs should have divided twice to generate four daughter cells and uninduced VPCs have divided once to generate two daughter cells. The average number of induced VPCs were calculated as the total number of induced VPCs divided by the number of animals observed. The average number of induced VPCs is in wild-type animals is three, and that in Hin animals is greater than three.

4. Identification and cloning of additional negative regulators

We have carried out several screens to obtain additional inhibitors of LET-23 - RAS signaling. In one screen, we seek mutations that cause RAS pathway activation (multivulva phenotype) in the absence of *sli-1* function. This screen identified *rok-1*, *rok-2* and several new candidates. In a second screen, we sought mutations that allow vulval formation when the LIN-3 growth factor is present at too low a level. This screen identified *rok-3* an X-linked semi-dominant suppressor of the *lin-3* vulvaless phenotype.

Mutations that synergize with *sli-1*. We have devised and initiated a new screen for additional genes that interact with *sli-1*. While mutations in a single negative regulator gene causes little or no vulval phenotype, in general, any combination of two or more of these mutants causes a synthetic multi-vulva (Muv) phenotype. A general strategy for identifying new negative regulators of vulval induction has involved the mutagenesis of a single mutant, such as *sli-1*, and screening for multivulva animals; this works but has a high background of *lin-2, 7* and *10* mutations. We reduced the frequency of *lin-2, lin-7* and *lin-10* mutations by overexpression of the receptor, *let-23*. Last year we demonstrated that this method is effective.

A screen of approximately 40,000 gametes was performed. Four Muv mutants were isolated that were not immediately recognizable as alleles of known genes conferring a visible multivulva phenotype on their own (*lin-1, lin-31, lin-15(null)*. In fact two of the five (*sy443* and *sy541*) do confer *sli-1*-independent Muv phenotypes and were not pursued further. We will try a *sli-1* and *sli-2* non-complementation screen as a final way to obtain genes of this type.

sy452 maps to the X chromosome. It fails to complement *rok-2 I* and *lin-15A* on *X*. We tentatively conclude that we are getting A class genes at low frequency in this screen. We will sequence to confirm that is indeed a *lin-15A* allele (*lin-15* locus encodes two transcripts from an operon). One implication is that *rok-2* is also an A class multivulva gene. We will pursue its molecular cloning in any case since it represents a new locus.

sy542 is being analyzed now to determine if it is worthy of pursuit.

rok-2(sy317) was isolated in the first round of screening in a *sli-1* background. We have mapped it to the left arm of chromosome I by standard linkage analysis (Brenner, 1974). *sli-1; rok-2* double mutants display an average of 4.2 VPCs generating vulval progeny per animal(n=22). *sli-2* partially suppresses a strong *sem-5* allele, *n1619*, and *let-23(sy1)*.

***lin-3* suppressors.** We discovered three new regulators of LET-23 signaling (previously referred to as *nrl-1, nrl-2 and nrl-3,)* as extragenic suppressor of the vulvaless phenotype of *lin-3*, which encodes the peptide ligand for LET-23. These have been tentatively renamed as *prl-1*, *rok-3* and *rot-1* after further analysis.

The *prl-1* (positive regulator of *lin-3*) mutation acts at or prior to LET-23. We do not yet know if it is los ofr gain of function based on genetic tests.

rok-3 acts at the level of LET-60 RAS: a *rok-3* mutation suppresses a *let-60 ras* but not a *lin-45 raf* mutation. We have discovered that it is a gain-of-function mutation, and thus *rok-3* is likely to be a positive regulator of LET-23 signaling.

genotype of suppressors	**genotype of *lin-3***	**vulva differentiation (VPCs/animal)**
+	+	3 (n>50)
+	*n378/n1059*	0.1 (n=20)
+	*n378*	0.58 (n=24)
prl-1(sy330)	+	3 (n=15)
sy330/+	*n378/n1059*	3 (n=4) (p<0.0001)
rok-3(sy340)	+	3 (n=23)
sy340/sy340	*n378/n1059*	2.73 (n=15)
sy340/+	*n378*	2.58 (n=6) (p<0.0001)
rot-1(sy341)	+	3 (n=18)
sy341/sy341	*n378/n1059*	2.6 (n=4) (p<0.0001)
sy341/+	*n378*	1.0 (n=19)
sy341/sy341	*n378*	2.84 (n=16)

Table 6: The phenotypes of *prl-1, rok-3, rot-1* and their effects on *lin-3*. n represents the number of animals examined. In several cases where the sample size is small, the p value from Fisher's Exact Test is shown. The p value of *n1059 prl-1(sy330)/n378 +* was calculated using *n1059/n378* as the control. The p value for *n378; rok-3(sy340)/+* was calculated using *n378* as the control. Control for *rot-1(sy341); n378/n1059* was *n378/n1059*.

rot-1 acts downstream of MAP kinase at the level of transcriptional regulators. The *rot-1(sy341)* mutation confers ligand-independent vulval development: 4 of 8 gonad-ablated animals had some vulval differentiation. *rot-1* maps close to *unc-4* on chromosome II based on three-factor mapping and failure to complement chromosomal deletions. Further deletion mapping will allow its molecular cloning this year. *rot-1* appears to act downstream of *lin-25* as indicated by its suppression of *lin-25* null mutation (Table 6).

suppressor genotype	genotype of vulva induction pathway	vulva induction (VPCs/animal)		
+	*let-23(sy97)*	0	(n=21)[a]	
prl-1(sy330)	*let-23(sy97)*	0	(n=27)	
+	*sur-1(ku1)*	1.9	(n=22)	
prl-1(sy330)	*sur-1(ku1)*	2.3	(n=22)	(p=0.1264)[b]
rok-3(sy340)	*let-23(sy97)*	2.9	(n=10)	(p<0.0001)
+	*let-60(n2034)*	0	(n=11)	
rok-3(sy340)	*let-60(n2034)*	2.3	(n=19)	(p<0.0001)
+	*lin-45(sy96)*	1.3	(n=10)	
rok-3(sy340)	*lin-45(sy96)*	0.4	(n=13)	(p=0.0058)
rot-1(sy341)	*let-60(n2034)*	2.6	(n=4)	(p<0.0001)
rot-1(sy341)	*lin-45(sy96)*	3.04	(n=12)	(p<0.0001)
+	*lin-25(e1446)*	0.97	(n=17)	
rot-1(sy341)	*lin-25(e1446)*	2.5	(n=13)	(p<0.0001)
+	*lin-39(n709ts)*	2.6	(n=35)	
rot-1(sy341)	*lin-39(n709ts)*	2.7	(n=41)	(p=1.0)[b]

Table 7. Spectrum of suppression analysis by *lin-3* suppressor mutations. Strains were constructed by standard methods (Brenner, 1974; Ferguson and Horvitz, 1985; Huang and Sternberg (1995).

5. Gene interactions

Most of the negative regulator mutations confer no phenotype on their own but are multivulva (resulting from pathway activation) in presence of another negative regulatory mutation. Lack of synergy of null mutations implies a functional interaction. A *sli-1* null mutation synergizes with a *gap-1* null mutation: while none of *sli-1* or *gap-1* single mutants are multivulva, 30% of the *sli-1 gap-1* double mutants are multivulva.

SLI-1 does not act through GAP-1, as rasGAP [Hajnal et al., 1997] synergizes with *sli-1*. Similarly, it is likely that ROK-1 does not act via GAP-1 because a *rok-1* mutation synergizes with a *gap-1* mutation.

genotypes	vulva induction (VPCs/animal)	% Muv
+	3.0 (n>50)	0
prl-1(sy330)	3.0 (n=15)	0
rok-3(sy340)	3.0 (n=23)	0
rot-1(sy341)	3.0 (n=18)	0
sli-1(sy143)	3 (n=20)[a]	0
rok-1(sy247)	3.03 (n=65)	3.1
rot-1(sy341); prl-1(sy330)	3.0 (n=35)	0
prl-1(sy330); sli-1(sy143)	3.0 (n=30)	0
sli-1 (sy143) rok-3(sy340)	3.0 (n=60)	0
rok-1(sy247); rok-3(sy340)	3.19 (n=48)	23 (p=0.0018)
rot-1(sy341); rok-3(sy340)	3.0 (n=29)	0
rot-1(sy340); sli-1(sy143)	3.88 (n=21)	48 (p=0.0005)
rot-1(sy341); rok-1(sy247)	3.29 (n=26)	27 (p=0.0019)

Table 8. *rok-3* and *rot-1* interact with other negative regulators of the vulva induction pathway. n represents the number of animals examined. % Muv are the number of animals with >3.00 VPSs generating vulva tissue. p values (Fisher's Exact Test) for double mutant strains with *rok-1* are calculated using *rok-1* alone as the control, and p values for double mutant strains with *sli-1* are calculated using *sli-1* alone as the control.

	lin-15B	*unc-101*	*sli-1*	*sli-2*	*rok-1*	*rok-2*	*rok-3*	*gap-1*
l +	0%	0	0	0	3%	0	0	0
in-15A	100%	0%	>0%	-	~50%	-	-	-
lin-15B		0%	0%	-	0%	-	-	-
unc-101			63%	-	29%	-	-	-
sli-1				10%	40%	77%	-	30%
sli-2					-	-	-	-
rok-1						83%	23%	90%
rok-2							-	-
rok-3								-

Table 9 Summary of interactions among negative regulatory mutants. % multivulva phenotype; 0%, Double mutant is wild-type; Muv, double mutant is at least partially multivulva; - not yet determined. All non-0 value are significantly different from 0%

6. Human homologs

We have designed and tested PCR primers of *rok-1* based on a comparison of ROK-1 and partial *Drosophila* sequence. We amplify several bands from a human cDNA library. These bands will be subcloned and sequenced to determine if they do represents human ROK-1 homolog(s). ROK-1 is similar in sequence to the human Ack protein and a partially characterized *Drosophila* cDNA encoding a tyrosine kinase. The *C. elegans* genome sequencing project has recently uncovered a *C. elegans* gene more similar to Ack than is likely a ROK-1 homolog in humans other

than Ack. We therefore have designed degenerate oligonucleotide primers corresponding to peptides conserved between ROK-1 and the *Drosophila* homolog. PCR reactions on DNA from a human cDNA library yielded several bands, which have been subcloned in preparation for sequencing to determine whether we have indeed cloned a fragment encoding a ROK-1 homolog.

7. Human cbl in *C. elegans*.

To test whether human cbl can function in *C. elegans* we had tried to rescue a *sli-1* mutation with h-cbl under the transcriptional control of a *C. elegans* inducible promoter. Since this was unsuccessful, we tested whether the highly conserved domain of h-cbl could substitute for the same domain of SLI-1. As shown in Figure 3, such a chimeric protein functions in *C. elegans*. Thus the conserved domain has conserved catalytic or protein-protein interaction functions. This suggests that studies of proteins that interact with this domain in *C. elegans* might be found in humans.

Conclusions

•**Mechanistic studies of SLI-1.** Functional domains of SLI-1 have been analyzed. A screen for interacting proteins has been carried out and four distinct candidate genes obtained and sequenced.

•***rok-1* encodes a novel protein tyrosine kinase.** The *C. elegans rok-1* locus, encoding a negative regulator of the LET-23 tyrosine kinase/LET-60 Ras pathway has been molecularly cloned. Extensive genetic and phenotype characterization of *rok-1* has been carried out. Search for a human homolog is underway.

•**New regulators.** New potential negative regulators have been identified and analyzed. *rot-1* acts at the level of the nucleus, while others (*sli-2*, *rok-2*) act on more upstream signaling events. *rok-3* now appears to be a positive regulator that acts at the level of RAS and RAF.

•**The conserved domain of human cbl functions in *C. elegans*.** A chimeric protein made from cbl and SLI-1 demonstrates the conserved nature of the amino-terminal domain of this family of proteins.

Progress by task as per original Statement of Work:

A brief description of progress on each task is listed.

Task 1A. Determine whether SLI-1 truncation decreases or increases activity of the protein as assayed in transgenic animals. •[Completed]

Task 1B. Determine role of alternative spliced form of SLI-1. •[Completed].

Task 1C. *sli-1* point mutation sequencing •[behind schedule; circumvented by site-directed mutagenesis studies].

Task 1D. *sli-1* antisera. We have obtained antisera that will be used to test for physical interactions of SLI-1 with candidate binding partners obtained from a two-hybrid screen in yeast. [on schedule].

Task 2A Genetic characterization of *sli-2*. [behind schedule]

Task 2B. Molecular cloning of SLI-2 from *C. elegans*. [behind schedule]

Task 3. Genetics and molecular cloning of ROK-1 from *C. elegans*. Final experiments for a comprehensive manuscript on *rok-1* genetics and molecular biology are in progress. [almost completed]

Task 4. Identification by genetic screens of new loci.

a. Screen for new mutations, carry out screens in parallel. [on schedule]

b. Genetic mapping and complementation of new mutations, parallel experiments • [on schedule]

c. Molecular cloning [on schedule]

Task 5. Examination interactions of genes in vivo [ongoing] •[on schedule]

Task 6. Human homologs. A search for a ROK-1 homolog has been initiated. •[on schedule/behind schedule]

Task 7. Introduction of c-cbl cDNA into transgenic nematodes. a. **Construct *sli-1/c-cbl* hybrid genes b. Examine phenotypes of transgenic animals.** •A chimera of c-cbl and SLI-1 functions in *C. elegans*; [completed].

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